Development and Characterization of Expressed Sequence Tag-Derived Microsatellite Markers for the Wheat Stem Rust Fungus *Puccinia graminis* f. sp. *tritici*

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ABSTRACT

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Puccinia graminis f. sp. *tritici* is the causal agent of stem rust disease in wheat. The rust fungus has caused devastating disease epidemics throughout history and is still posing a potential threat to wheat production in some regions of the world due to the appearance of new races. To develop microsatellite or simple sequence repeat (SSR) markers for use in population genetics studies, a total of 60,579 expressed sequence tag (EST) sequences (reads) generated from *P. graminis* f. sp. *tritici* were screened for tandemly repeated di- and tri-nucleotide units using a bioinformatics approach and 708 unisequences containing putative SSR loci with six or more repeat units were identified. Flanking primers were designed for 384 unique SSR loci, which mapped to different locations of the draft genome sequence of the fungus. Of the 384 primer pairs tested, 72 EST-SSR markers were eventually developed, which showed polymorphism among 19 isolates of *P. graminis* f. sp. *tritici* and 4 isolates of *P. graminis* f. sp. *secalis* evaluated. Thirty-two of the SSR loci were also evaluated in three other rust fungi (*P. triticina*, *P. hordei*, and *P. coronata* f. sp. *hordei*) for cross-species transferability. These SSR markers derived from ESTs will be useful for characterization of population structures and for gene mapping in *P. graminis*.

Wheat stem rust, caused by *Puccinia graminis* f. sp. *tritici*, has been one of the most devastating diseases in human history. In the early to mid 1950s, stem rust epidemics caused approximately 50% yield losses of wheat in North Dakota and Minnesota due to the emergence of a new race (race 15B) (21,22). Since 1974, stem rust has been under control in North America due to the incorporation and pyramiding of stem rust resistance genes into wheat cultivars. The barberry (*Berberis vulgaris*) eradication program, initiated in the early 1920s, also maintained the effectiveness of resistant genes longer by reducing the chance of the generation of new virulent races through sexual recombination of the fungus (22). However, wheat stem rust still poses a potential threat to wheat production worldwide. Especially, a new race (Ug99 or TTKS) and its variants were recently found in Eastern Africa and caused serious disease problems in that region (26,34). They have also triggered a serious concern to many other regions of the world where the stem rust resistance genes deployed in commercially grown wheat varieties were not effective against them (13–15).

P. graminis f. sp. *tritici* is a heteroecious macrocyclic fungus with five spore stages (22). The uredia and telia are produced on wheat while the pycnia and aecia are formed on the alternate host, barberry (*B. vulgaris*). The uredinial stage is the most prevalent stage of the life cycle and can repeat on gramineous hosts under favorable conditions. In the late season, the rust pustules form telia, which produce teliospores. Meiosis occurs in teliospores and basidiospores emerge after teliospores germinate. Basidiospores can infect barberry plants and produce pycnia with pycniospores and receptive hyphae. The receptive hyphae from one mating type can be fertilized by a pycniospore of opposite mating type to produce aecia with dikaryotic aeciospores. The aeciospores infect cereals and reproduce urediniospores again. Significant variation within a *P. graminis* f. sp. *tritici* population can be generated through genetic recombination during this sexual cycle when barberry plants exist, although several other mechanisms also operate, such as mutation, somatic hybridization, and alien introduction (5). Virulence tests are commonly used to detect pathogen variation and a number of races have been identified. However, virulence markers are limited and subjected to host selection. Since the 1980s, molecular markers have been used to study the genetic variation and population structure of fungi, including *P. graminis* f. sp. *tritici*. Using enzyme markers combined with virulence evaluation, Burdon and Roelfs (6) compared the sexually and asexually reproducing populations of *P. graminis* f. sp. *tritici* and found that the sexually reproducing population exhibited higher genetic diversity compared with the asexually reproducing population. Other markers including randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) were also used to analyze genetic diversity in wheat stem rust (24) and other rust fungi (8,20). Although these markers provide powerful tools for population genetic studies, each of them has limitations in application. For the dikaryotic organisms like *Puccinia* species, codominant markers such as SSRs are more informative in revealing genetic variations when compared to dominant markers (30).

SSR markers have been developed for many species of plants and fungi and are usually developed from genomic DNA through the construction of SSR-enriched libraries. This approach, although effective, is quite time consuming and labor-intensive. In recent years, more and more expressed sequence tags (ESTs) from cDNA libraries have been generated for a number of organisms,

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including fungi. These ESTs have served an important resource for developing new SSR markers to assess genetic diversity. EST-SSRs have been previously developed and characterized from a number of plant species (9,17,33), as well as from fungal species (10,11). In *P. graminis* f. sp. *tritici*, EST-SSRs were not available although 25 SSR markers were developed from genomic DNA clones of SSR-enriched libraries (32). To develop additional SSR markers, we screened 6,0579 EST sequences of *P. graminis* f. sp. *tritici* and developed 72 EST-derived SSR markers. These additional SSR markers were characterized using various isolates of *P. graminis* f. sp. *tritici* and other taxa of rust fungi.

MATERIALS AND METHODS

Fungal isolates. Nineteen isolates of *P. graminis* f. sp. *tritici* and four isolates of *P. graminis* f. sp. *secalis* were used in the study (Table 1). In addition, one isolate from each of the three *Puccinia* species, i.e., *P. triticina*, *P. hordei*, and *P. coronata* f. sp. *hordei*, provided by J. Rasmussen, B. Steffenson, and Y. Jin, respectively, was used to test cross-species amplification of the EST-SSR primers developed from *P. graminis* f. sp. *tritici*. Isolates of *P. graminis* f. sp. *tritici* were increased on the seedlings of the susceptible variety 'Little Club' (CItr 4066) and those of *P. graminis* f. sp. *secalis* were increased on the susceptible rye accession 'Prolific' (PI 535177). The seedling plants used for rust increase were treated with malaic hydriate before inoculation to enhance spore production (29). Thirteen of the *P. graminis* f. sp. *tritici* isolates were previously characterized for virulence on a set of standard stem rust differentials by Sun and Steffenson (31) and a four letter code was assigned to them based on their infection reactions on the differentials (27). The other *P. graminis* f. sp. *tritici* isolates were evaluated on the same set of differentials following the same procedure as described by Sun and Steffenson (31).

Generation of EST sequences and identification of microsatellites. Three cDNA libraries were constructed from urediniospores, germinated urediniospores, and teliospores of the *P. graminis* f. sp. *tritici* isolate 7A (CRL 75-36-700-3), respectively (37). These cDNA libraries were sequenced by the Broad Institute, MIT and 60,579 EST sequences were generated. Assembling of the ESTs into contigs and singlets was performed using the CAP3 program (12). The resulting unique sequences were screened for microsatellite loci with di- and tri-nucleotide combinations tandemly repeated six or more times using a Perl script provided by Zheng Jin Tu at the University of Minnesota, St. Paul. Sequences containing microsatellites were analyzed by BLASTX (1) to identify similarities to previously characterized sequences in GenBank and thereby deduce possible gene function. The BLASTN search was also performed to map these sequences into the supercontigs and contigs assembled from the genomic sequences generated from the isolate 7A of *P. graminis* f. sp. *tritici*.

Primer design, polymerase chain reaction (PCR) amplification and polymorphism evaluation. Primers were designed within the flanking regions of each microsatellite locus using Primer3 with the default settings: OPT_SIZE = 20; MIN_SIZE = 18; MAX_SIZE = 27; MIN_TM = 57; and MAX_TM = 63. In order to generate PCR products with fluorescent-labeled M13 primer incorporated, an M13 tag (5′-CACGACGTTGTAAAACGAC) was added to the 5′ end of each forward primer. Primer pairs designed were initially evaluated for amplification using DNA from the isolate QCC-2. PCR was performed in an MJ Research PTC-100 thermal cycler (Watertown, MA) with the following profile: 95°C for 5 min, 3 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 80 s, 33 cycles at 94°C for 15 s, 52°C for 15 s, and 72°C for 45 s, followed by a 4°C holding step. Each PCR amplification contained 1× PCR buffer (10 mm of Tris-HCl, 50 mm of KCl), 200 µm of dCTP, dGTP, dTTP, and dATP, 1.5 mm $MgCl_2$, 5 pmol M13 primer labeled by IRD700 or IRD800 (LI-COR, Lincoln, NE) at the 5′ end, 1 pmol 5′-tagged forward primer, 5 pmol reverse primer, 1 U of *Taq* polymerase (New England Biolabs, Ipswich, MA) and 10 ng of rust genomic DNA in a final volume of 10 µl. The PCR products were diluted 10- to 20-fold and analyzed on an LI-COR 4300 DNA sequencer (Lincoln, NE) using a 7% polyacrylamide gel. Primers that gave clear amplicons were further evaluated on 19 isolates of *P. graminis* f. sp. *tritici* and 4 isolates of *P. graminis* f. sp. *secalis* for polymorphism analysis. SSR primers shown to be polymorphic among the *P. graminis* isolates were also evaluated on one isolate of *P. hordei*, *P. triticina* and *P. cronata* f. sp. *hordei* to test the

TABLE 1. Rust isolates used for characterization of microsatellite markers developed from *Puccinia graminis* f. sp. *tritici* expressed sequence tags

Species/subspecies	Isolate	Pgt code[a]	Provider
Puccinia graminis f.sp. *tritici*	CRL-1	HKHJ	B. Steffenson
	CRL-2	HPQJ	B. Steffenson
	CRL-3	RKQQ	B. Steffenson
	CRL-4	HTMJ	B. Steffenson
	CRL-6	RCRS	B. Steffenson
	CRL-7	TPQK	B. Steffenson
	CRL-8	TCQK	B. Steffenson
	MCC	MCCF	B. Steffenson
	QCC-2	QCCJ	B. Steffenson
	A-12	TCTS	B. Steffenson
	ORGE	JCCJ	B. Steffenson
	WM-1	RCCD	B. Steffenson
	36-55a	RCHN	B. Steffenson
	Gb-121	JCMN	B. Steffenson
	111	LCBL	B. Steffenson
	72-22	RHTS	B. Steffenson
	HJCS	HJCS	B. Steffenson
	4A (CRL 78-21-BB463)	DFBJ	L. Szabo
	7A (CRL 75-36-700-3)	SCCL	L. Szabo
P. graminis f.sp. *secalis*	92-MN-90	–	B. Steffenson
	HQ	–	B. Steffenson
	KR	–	B. Steffenson
	HT	–	B. Steffenson
P. triticina	THBL	–	J. Rasmussen
P. hordei	Race 8	–	B. Steffenson
P. cronata f.sp. *hordei*	ND91-36	–	Y. Jin

[a] The Pgt code is based on the reaction on a set of 16 stem rust differentials (27). The – indicates data were not applicable.

transferability. Polymorphism information content (PIC) for each EST-SSR locus was calculated using the online program. All DNA samples used in the study were extracted from urediniospores using a FastDNA kit (MP Biomedicals, Solon, OH) and a FastPrep Instrument (MP Biomedicals, Solon, OH) according to the manufacturer's protocol.

Genetic diversity analysis. The alleles of each SSR locus amplified from individual isolates of *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *secalis* were scored and designated as 0 or 1 according to the absence or presence of the allele. Genetic similarity between isolates was measured as coefficients using the SimQual program of NTSYSpc version 2.021 (Exeter Software, Setaukal, NY). The unweighted pair group method of arithmetic averages (UPGMA) (SAHN program, NTSYSpc version 2.021) was used to construct a phenogram representing genetic similarities based on the SSR data. To estimate the strength of the groupings generated by the cluster analysis, bootstrap analysis was conducted with 500 replications using the Winboot computer program (25).

RESULTS

Identification of SSRs in EST sequences. The 60,579 EST sequences generated from three cDNA libraries of *P. graminis* f. sp. *tritici* (37) were clustered into 11,853 unique sequences, which consisted of 4,184 contigs and 7,669 singletons. A database was constructed for these unique sequences and used to search for the 14 unique classes of di- and tri-nucleotide repeats described by Jurka and Pethiyagoda (16) and Katti et al. (19). A total of 708 unique sequences (contigs or singletons) were found to contain microsatellites with repeat numbers equal to or greater than 6. The number of repeat units ranged from 6 to 14, but the majority (75%) of the microsatellites had six or seven repeat units. All 14 unique classes of di- and trinucleotide repeats were found, but the frequencies of the classes varied among the 708 microsatellites (Fig. 1). Class 1 (AT/TA), 2 (AG/GA/CT/TC), and 3 (AC/CA/TG/GT) were the most frequent of the dinucleotide classes, which had 68, 218, and 120 occurrences, respectively. Class 6 (AAG/AGA/GAA/CTT/TTC/TCT), 7 (AAG/AGA/GAA/CTT/TTC/TCT), and 8 (ATG/TGA/GAT/CAT/ATC/TCA) were the most frequent trinucleotide classes, which had 50, 99, and 64 occurrences, respectively. The remaining classes were found from 3 to 21 times each (Fig. 1).

To map the microsatellite loci to the draft *P. graminis* f. sp. *tritici* genome sequence, BLASTN searches were conducted against the 4,557 contigs in 392 scaffolds (supercontigs) assembled from the genome sequence reads of *P. graminis* f. sp. *tritici*. All of the 708 microsatellite loci were mapped onto contigs based on their BLASTN matches at an E-value of 1×10^{-25} or less. We selected 414 of the loci for further primer design based on their location and distribution on contigs and supercontigs of the *P. graminis* f. sp. *tritici* genome. BLASTX searches against the GenBank database were conducted with these 414 sequences. However, most of them did not have matches or had similarity to sequences with no useful annotation (e.g., hypothetical or unnamed protein) in the database when an E-value of 1×10^{-5} was used as cutoff (data not shown). Among the 72 microsatellite loci listed in Table 2, only seven had matches with meaningful annotation (data not shown).

Amplification and polymorphism. Primer pairs were successfully developed for 384 of the 414 microsatellite loci selected above. For the remaining 30 microsatellite loci, primer design was unsuccessful either because the repeats were too close to an end of the sequence or the flanking sequences did not meet the parameters set for primer design. The 384 primer pairs were initially screened for amplification on the DNA sample isolated from the isolate QCC-2. Only 202 of the primer pairs showed good amplification. These 202 primer pairs were further evaluated on 19 isolates of *P. graminis* f. sp. *tritici* and 4 isolates of *P. graminis* f. sp. *secalis* for polymorphism. Only 72 gave clear, easily scored, polymorphic banding patterns across at least 20 of the 23 isolates (Fig. 2). These 72 primer pairs were considered useful and were included in Table 2. The remaining 130 were not included in Table 2 because they either didn't show polymorphism, or gave amplification in some isolates, but not in others, or the bands amplified by them were too numerous to be resolved, or not of the expected size.

Among the 72 primer pairs shown in Table 2, 53 were polymorphic between the two isolates 4A (CRL 78-21-BB463) and 7A (CRL 75-36-700-3), which were used to make a mapping population for the development of a partial genetic map of *P. graminis* f. sp. *tritici* (36). An additional 46 primer pairs were also found to be polymorphic between isolates 4A and 7A when the remaining 130 primer pairs were evaluated (data not shown). This gave a total number of 99 primer pairs exhibiting polymorphism between these two parental isolates.

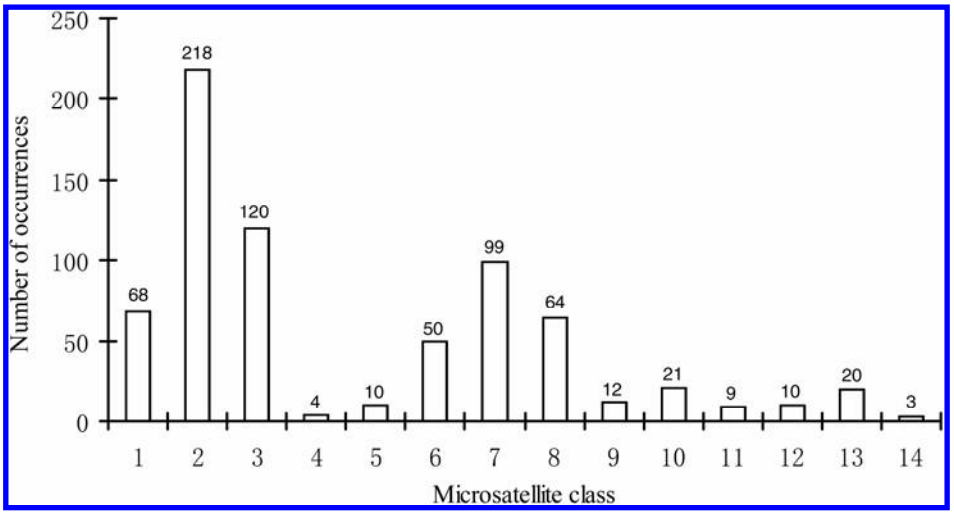

Fig. 1. Frequency distribution of the 14 unique classes of di- and tri-nucleotide repeats (16,19) among 708 potential microsatellite loci identified from the expressed sequence tag database of *Puccinia graminis* f. sp. *tritici*. Microsatellite classes are as follows: class 1 = AT/ TA; class 2 = AG/GA/CT/TC; class 3 = AC/CA/TG/GT; class 4 = GC/CG; class 5 = AAT/ATA/TAA/ATT/TTA/TAT; class 6 = AAG/AGA/GAA/CTT/TTC/TCT; class 7 = AAC/ACA/CAA/GTT/TTG/TGT; class 8 = ATG/TGA/GAT/CAT/ATC/TCA; class 9 = AGT/GTA/TAG/ACT/CTA/TAC; class 10 = AGG/GGA/GAG/CCT/CTC/TCC; class 11 = AGC/GCA/CAG/GCT/CTG/TGC; class 12 = ACG/CGA/GAC/CGT/GTC/TCG; class 13 = ACC/CCA/CAC/GGT/GTG/TGG; class 14 = GGC/GCG/CGG/GCC/CCG/CGC.

Genetic diversity. A total of 359 polymorphic alleles were generated from the 72 SSR loci across 19 isolates of *P. graminis* f. sp. *tritici* and 4 isolates of *P. graminis* f. sp. *secalis*. The number of alleles ranged from 2 to 10, with an average of 5 alleles per locus. The presence or absence of an allele in each isolate was scored as 1 or 0, respectively. Distance metrics were calculated, and a UPGMA dendrogram was derived to assess genetic diversity among the 23 isolates of *P. graminis* (Fig. 3). The resulting phenetic dendrogram showed that 22 of the 23 samples had distinct genotypes. Isolates CRL-2 and CRL-4 had the same genotypes based on the SSR loci evaluated. However, these two isolates showed different virulence phenotypes (Table 1); they could be derived from the same lineage but with mutations at the virulence loci (5). Two (92-MN-90 and HQ) of the four *P. graminis* f. sp. *secalis* isolates formed a cluster, which were separated from other *P. graminis* f. sp. *tritici* isolates. The other two rye stem rust isolates (KR and HT) were clustered with several other *P. graminis* f. sp. *tritici* isolates (CRL-2, -3, -4, -7, and -8) and appeared to be more closely related to *P. graminis* f. sp. *tritici* than to *P. graminis* f. sp. *secalis*. Other small clusters were also identified, for example, CRL-1 and HJCS, 36-55a and 4A, 72-22 and CRL-6, and A-12. These groupings were supported by the bootstrapping analysis with a bootstrap value >70 (Fig. 3).

Cross-species amplification. To test the transferability of the EST-SSR primers developed from ESTs of *P. graminis* f. sp. *tritici* to other rust species, 32 of the 72 primer pairs were used for PCR with DNA templates from one isolate of *P. triticina*, *P. hordei*, and *P. cronata* f. sp. *hordei*, respectively. The results showed that nine of the 32 primer pairs produced amplicons in the *P. triticina* isolate THBL and nine primer pairs in race 8 of *P. hordei* (Table 2). Seven of the primer pairs also amplified DNA fragments from the barley crown rust isolate ND91-36 (Table 2).

DISCUSSION

Our results indicated that the EST sequences generated from *P. graminis* f. sp. *tritici* provided a rich source of additional microsatellite markers for population genetics studies in the fungus. Among the 11,853 unique sequences (including contigs and singletons) assembled from a total of 60,579 EST sequences, approximately 6% (708/11853) contained microsatellites. The discovery rate is higher than that reported for other fungi, which have di- or tri-nucleotide microsatellites in 1.3 to 4.4% of the EST sequences (11). The reason for the high number of microsatellite-containing ESTs in *P. graminis* f. sp. *tritici* is not known. *P. graminis* f. sp. *tritici* has a larger genome size compared to most other fungi that have been sequenced, but this is probably not the major reason for the high occurrence of SSRs in the EST database because the genome size is neither inversely nor directly proportional to the SSR abundance as has been reported for other genomes (18). It is possible that the dikaryotic nature of the fungus may contribute to the high number of SSRs in the EST sequences because the expansion of SSRs is more tolerated in diploid organisms than in haploid fungi (35). Lim et al. (23) compared the abundance of microsatellites in 14 fungal genomes and also found that *Candida albicans*, the only diploid fungus included in the study, possesses higher number of mono- to hexanucleotides than any other fungal genomes analyzed.

Among the 708 microsatellites, 410 were dinucleotide and 398 were trinucleotide repeats. The result is in contrast to those that have been reported previously in EST databases from other organisms (17,33) where trinucleotide microsatellites are more abundant. The most commonly identified individual microsatellite in the *P. graminis* f. sp. *tritici* EST database was the dinucleotide $(AG)_n$, followed by $(AC)_n$. A high frequency of $(AG)_n$ and $(AC)_n$ microsatellites was also reported for plants (17,33) and other fungi (11,18,23). The $(AT)_n$ microsatellite class was the first- or second-most frequent in most other fungi (18,23) while they were the third-most frequent class in our *P. graminis* f. sp. *tritici* ESTs. However, the frequency of $(AT)_n$ microsatellites in the *M. graminicola* EST database was low (11). For the trinucleotide microsatellites, differences in frequency were also observed between the *P. graminis* f. sp. *tritici* database and those for other fungi. For example, $(AAC)_n$ was the most common trinucleotide repeat in the *P. graminis* f. sp. *tritici* database, but occurs less common in several other fungal genomes (18,23). $(CCG)_n$ was the least common microsatellite in the ESTs of *P. graminis* f. sp. *tritici* and was the case for most other fungal genomes (18,23), but it was the most frequent in *Magnaporthe grisea* (18) and moderately common in *M. graminicola* (11). Also, $(CCG)_n$ microsatellites were the most common in several plant species, including barley (33) and sugarcane (9). All these results indicate that the relative abundance of each class of SSRs varies with different organisms although some similarity exists, as has been suggested by Lim et al. (23) and Karaoglu et al. (18).

We initially evaluated 384 primer pairs for PCR amplification with the DNA isolated from the isolate QCC-2, but failed to generate amplicons with 182 of the primer pairs after at least two attempts. There were several possible reasons for the failure of PCR amplification from the EST-SSR loci. First, for simplicity and convenience, we used the same annealing temperature (52°C) in PCR for all the primers. This might be suitable for some primers but not for others. Optimization of PCR conditions may improve amplification efficiency. Second, the primers were designed based on EST sequences. Introns or other structures in the genome may have prevented amplification. When the introns were too long or the primers were designed from the adjoining regions of an intron, no amplification would occur. Comparison of the EST-SSR loci with the sequenced genome sequence indicated that some of the failed primers flanked a large intron (>1.5 kb) or mapped to the region where there was no sequence match (data not shown). Dracatos et al. (10) provided another evidence of the presence of introns in a number of EST-SSR loci of the crown rust fungus (*P. coronata* f. sp. *lolli*) by sequence analysis. Third, since there was heterogeneity between the dikaryotic nuclei of the urediniospore and the ESTs were generated from the isolate 7A used for the whole genome sequencing, point mutations, or insertions and deletions within the priming sites could also account for PCR failure in other isolate (i.e., QCC-2). We aligned the primer sequences to the genomic sequence of isolate 7A and found the majority of the failed primer (s) showed 1 to 4 bp mismatch to the genomic DNA (data not shown).

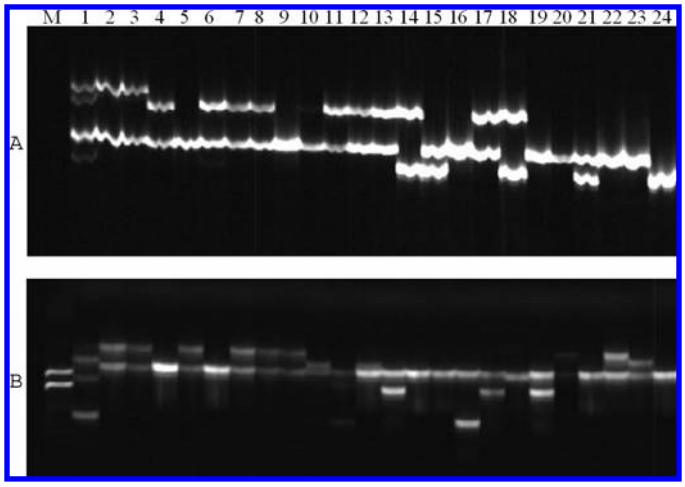

Fig. 2. Polymorphism revealed by the expressed sequence tag derived microsatellite markers, Pgestssr161 (**A**) and Pgestssr269 (**B**), among four isolates of *Puccinia graminis* f. sp. *secalis* and 20 isolates of *P. graminis* f. sp. *tritici*. M. 100-bp DNA ladder. No. 1, 10, 11, and 12 are *P. graminis* f. sp. *secalis* isolates and the others are *P. graminis* f. sp. *tritici* isolates.

TABLE 2. Information of the 72 expressed sequence tag (EST)-simple sequence repeat (SSR) markers identified from the EST sequences of *Puccinia graminis* f. sp. *tritici*

Locus[a]	Location in the genome[b]	Forward primer sequence	Reverse primer sequence	Tm[c]	Size[d]	Class[e]	Motif	No. of repeats	No. of alleles	PIC[f]	Cross-species amplification[g]
Pgestssr015*	Supercontig 2: 778229-778453	ATAAAGCGCGTGACCAATCT	CCGCTGAATTCCTTCATGTT	60	227	1	AT	6	5	0.7029	–
Pgestssr026*	Supercontig 3: 167011-167257	AGCCCCAACTGCAGAACA	CGAAGCTTGAGAGGAGGTTG	60	247	3	AC	7	5	0.6176	–
Pgestssr029*	Supercontig 3: 1735447-1735636	TATAGCCGCGTCCAAGGATA	TTCGAGTTGAGTGTCGATGC	61	190	2	AG	9	3	0.3856	–
Pgestssr039*	Supercontig 4: 1215971-1216204	CAAATTTTCATCTTTTTCGTCTTG	GGCATTCTCGTGATCAACCT	59	235	1	AT	8	5	0.6759	–
Pgestssr047*	Supercontig 4: 244727-244962	AATCTCATCCAATCGCCAAC	CTTGGTCTTGACGGAGAAGG	60	236	10	AGG	6	3	0.5197	–
Pgestssr091*	Supercontig 9: 1038855-1039050	TTCGAGCTGTGAAGACGTTG	TGAACAGCCAGTTGATGAGG	60	196	11	AGC	8	4	0.6086	–
Pgestssr098*	Supercontig 10: 736629-736823	CAGTGGGAGGGAGAATAACG	GCCTCTTCGAAGTTGTTGCT	60	195	2	AG	14	7	0.7256	–
Pgestssr101*	Supercontig 11: 1253476-1253632	CTCAACCGCAATAACAGCAA	GCGACCCAATCATGAATCTT	60	157	2	AG	7	8	0.8160	–
Pgestssr103*	Supercontig 12: 218500-218658	GCCACAGGGGATACTTTTAGC	GAACCGGAAGAGACCAATGA	60	159	2	AG	7	6	0.7232	–
Pgestssr109*	Supercontig 13: 54732-54901	CCATCCGATCATTTCTTCGT	CCGACCTTCTCTTGCTTCTG	60	176	10	AGG	7	5	0.7043	–
Pgestssr121	Supercontig 14: 685609-685782	CGGAAAATTGAGGGAAGACA	CGGCGTCCTAGAAACAGAAC	60	174	2	AG	7	7	0.7829	–
Pgestssr124*	Supercontig 14: 842917-843156	TCCTGACATGCAATTTGGTT	GAGCCTAACAATCCCCACAA	59	242	2	AG	12	7	0.7499	–
Pgestssr129*	Supercontig 15: 243999-244226	TGGAGCCTTGAGGAAGTGAT	CCAAAGACGAGGTGGAAAAG	60	230	2	AG	7	6	0.7264	–
Pgestssr130*	Supercontig 15: 49956-50160	ACAGGATGCTTGGGTTGAAG	CGCTCAGCCAGTTTTTCTTT	60	205	3	AC	13	6	0.7723	–
Pgestssr131*	Supercontig 15: 285965-286199	AGCTGGGGGAAACAAAAGTT	GACCATTCCATCCATCGTTT	60	235	3	AC	6	10	0.7790	–
Pgestssr139*	Supercontig 16: 1157322-1157516	TGCGTTTAGGAGCAGGTTTT	GTCAAACCACCTCGACGACT	60	195	11	AGC	6	4	0.5928	–
Pgestssr149*	Supercontig 18: 479864-480074	GGGGAGAAGCACAATCACAT	CGGTTCCCAATGACAAAAAC	60	213	2	AG	7	7	0.7730	–
Pgestssr157*	Supercontig 18: 761350-761563	AAGAACCTTCGAGCAGTCCA	CGATTCGGTGTAGGCTGATT	60	214	8	ATG	6	4	0.4498	–
Pgestssr161*	Supercontig 19: 313252-313489	TGAGGAGACGATTTTTGAGAA	TAGAGAAGGAGGGCAAGGTG	58	238	2	AG	6	4	0.6072	–
Pgestssr165	Supercontig 20: 792415-792627	CATCTTGAGTTGGCCATTTG	TCCGAAGACTTTCTTGAATCC	59	213	3	AC	7	5	0.6046	–
Pgestssr171	Supercontig 21: 684080-684277	GGTTTGGGGAACTGATTCAA	AATCCAACCCAAACCATTCA	60	198	11	AGC	7	4	0.5501	–
Pgestssr173	Supercontig 22: 710658-710839	TCCCTTGACCTTTCTCAACG	TCCATTGAGTTCCATCGTGA	60	181	8	ATG	6	5	0.7310	–
Pgestssr185*	Supercontig 24: 145241-145404	AGCGACCGACGAGGAGACT	GTCCCTCGTCCTCCTCAGAT	63	164	8	ATG	6	3	0.4763	–
Pgestssr194*	Supercontig 26: 692315-692546	GGGGGATAAGGAAACCAGAT	ATCTCTGGCCACTCGGTATG	59	235	3	AC	6	7	0.6827	–
Pgestssr196*	Supercontig 27: 142460-142665	TGACGATGATCCAGAAGCAG	TGGGAAGGGAAGTTTGACTG	60	218	8	ATG	6	9	0.7965	–
Pgestssr206*	Supercontig 28: 75400-75640	CATACTCCACTACCACTTCACCA	GGGATCGAGTTCTGTTCGAT	59	176	3	AC	9	4	0.5254	–
Pgestssr207	Supercontig 28: 472127-472375	GGAAAGGACAATGTCAATCTGG	TGGCGAAAATCAGCTTGC	61	249	3	AC	8	6	0.7583	–
Pgestssr210*	Supercontig 29: 685596-685817	TGCACCACGATAATTGCTGT	AACAGCTCAACCAGGACCTC	60	227	2	AG	8	4	0.5037	–
Pgestssr226	Supercontig 33: 82830-83075	GGAAAAGGGGGAAAGAAAAA	CTGCCGGTTGACTCCTTG	59	249	1+2	AT+AG	7	4	0.5797	–
Pgestssr227	Supercontig 33: 496809-497010	CACACGTCTCGAGGAACAGA	CTCGTGGGATGAAGTCCATT	60	202	6	AAG	11	5	0.6715	–
Pgestssr231*	Supercontig 35: 410292-410496	TGAAAGCGAAAACTTCACACA	ACGACCCATCAAAAACAAGC	60	214	6	AAG	7	7	0.6938	–
Pgestssr232*	Supercontig 35: 846698-846861	TAGAGACCCTCCCCAAATCC	ACGAAGAGTTTGACCCTGGA	60	172	1	AT	7	5	0.6853	–
Pgestssr235	Supercontig 35: 382307-382696	CAAACCTTGCATGGATGAGA	AGCGGGAGCTCACATAACTG	60	221	1	AT	6	3	0.5392	–
Pgestssr237*	Supercontig 35: 558664-558913	TGGTGAACAATAGCAACCACA	TTCCTTTTCTTCTCTTATTGTTGG	60	246	6	AAG	7	5	0.7034	–
Pgestssr243*	Supercontig 39: 440951-441180	GCTGCGTCGACTAATCCTTC	ATCAGCACCAGTAGCCGAAT	60	224	2	AG	8	4	0.6035	–
Pgestssr244*	Supercontig 39: 400036-400216	GAAGCTCCAAGGCAGAAATG	AGGAGTGGGTCAGGATGATG	60	181	6	AAG	7	5	0.6813	–
Pgestssr250*	Supercontig 41: 653488-653650	CCGGTCATAAACCGAGAATG	GAAGGTCCAGCCTTCGTGTA	60	163	6	AAG	7	4	0.4611	–
Pgestssr251	Supercontig 41: 310866-311016	CCAAGCAAAGGAGAAGAGGA	TTCTTGGAACTCGGTTTTCG	60	153	2	AG	6	3	0.4526	–
Pgestssr260*	Supercontig 43: 324448-324604	TGGAAAACGATCAAACCACA	TGTTCGATTGGGAAGAGTCC	60	157	12	ACG	8	2	0.3457	–
Pgestssr269*	Supercontig 46: 552369-552547	TGGTTTGTTTGTGGTGATGG	TCGCTCCATACTTTCCTTCG	60	187	2	AG	8	7	0.5851	–
Pgestssr270*	Supercontig 46: 320140-320308	GGAGTTGTCGCACCTGTCTT	GTCTGCCGCAGTTTTCTCTT	60	170	8	ATG	6	4	0.6218	No Amplicon

Pgestssr271*	Supercontig 46: 344313-344536	CAGGCCTTAAAGTCCAACCA	CCAGGTACCACGATCAAACC	60	227	3	AC	7	5	0.5986	No Amplicon
Pgestssr273	Supercontig 47: -	CGTCCTCTTCTTGGACTTGC	AATGACCCGCATAGTCATCC	60	209	1	AAC	7	7	0.6875	No Amplicon
Pgestssr278*	Supercontig 48: 459098-459300	CACGCTGACTTGATGAGAGC	CATAAGGCCTGGATTCCGTA	60	218	3	AC	6	4	0.6525	Pt
Pgestssr279	Supercontig 48: 120281-120467	ATCGAAGAGCCGTTCACTGT	AGGGAATCCGATCAAGGAGT	60	187	8	ATG	6	4	0.3968	No Amplicon
Pgestssr281*	Supercontig 49: 368863-369038	ATGAGCAAATGACTGCAGGA	GCCTCATCCTTCCAATCAAG	60	194	8	ATG	6	3	0.4598	No Amplicon
Pgestssr283	Supercontig 51: 387848-388021	TGTCCTCTGACTTTGGCTTG	CAGCTTTCTTCACCACGATG	59	174	2	AG	6	4	0.6992	No Amplicon
Pgestssr288*	Supercontig 52: 130153-130378	ACAGGACTTGCCAGGAAGAA	GGGCCTGATGACTCTGTTGT	60	223	2	AG	6	6	0.6922	No Amplicon
Pgestssr293	Supercontig 54: 507344-507589	GAACCTTGGCCTGAGTGCTA	GCAGCCTACAGCAAGAATCC	60	249	13	ACC	6	5	0.5909	Pt
Pgestssr294*	Supercontig 54: 131909-132071	ACCTGGCCATCAAGAATCTG	GGAGGCACGGTCATAAGAAA	60	175	10	AGG	7	3	0.4140	Ph
Pgestssr303	Supercontig 57: 338375-338565	GTTGTCGATCAGTCGAAGCA	CGTCTCCCATCAGGACATCT	60	191	6	AAG	6	4	0.4620	Ph, Pch
Pgestssr311*	Supercontig 60: 63194-63427	ACGCCAGTTTTTGGTCAATC	CGTCGTCTTGGACGAGTTCT	60	232	2	AG	8	8	0.8274	Pt, Pch
Pgestssr329*	Supercontig 73: 225021-225186	AGCCAAAGGTTGATTGTTGG	GTTTCCAGACGAGGAAGACG	60	166	8	ATG	6	7	0.6570	No Amplicon
Pgestssr332*	Supercontig 74: 120673-120913	GATCACTGGCTTCCTCGAAC	GCAAGGAATCCAAGACCTGA	60	241	8	ATG	7	4	0.693	Pt, Ph
Pgestssr337	Supercontig 80: 128757-129096	CGAGATGTCAGTGGAAGCAA	CAGCCTGAAGGAACTCGAAC	60	245	3	AC	6	2	0.3146	Pt
Pgestssr341*	Supercontig 83: 226991-227207	GATGTCGCACTCGGTTTCTT	GGCCTTGGTACCCAATTTCT	60	217	13	ACC	6	2	0.3684	No Amplicon
Pgestssr345*	Supercontig 86: 25980-26176	TGGTGCCAGTCTATTGATCG	TGGATTGGATGCCTTTTAGG	60	215	9	AGT	13	6	0.575	No Amplicon
Pgestssr346*	Supercontig 87: 221314-221492	TGTCAATAAATAAACAATCAAAAGCA	GGAAAGATGGTGTAAGGAGGAA	59	179	2	AG	10	4	0.6354	Pt, Ph, Pch
Pgestssr350*	Supercontig 90: 252294-252500	TGCCAGTTCTGTTCATCAGC	TTGAAAGGAGGTGGAGGATG	60	207	8	ATG	6	5	0.6341	No Amplicon
Pgestssr353*	Supercontig 92: 168751-168991	TCGAATCCCAAGGAACAGAG	ACGTCTTGGGTTTCTGTGGA	60	241	10	AGG	6	4	0.5501	No Amplicon
Pgestssr355	Supercontig 94: 211869-212036	ACCTATTCATTTCCCCATCG	GGAAAGTTCACCGAAAAGCA	59	170	1	AT	6	6	0.6554	Ph, Pch
Pgestssr358	Supercontig 95: 199546-199737	CGAGGGATAATACGGCAAGA	CCCGACCAGTCTTCATGATT	60	194	6	AAG	6	4	0.6338	Pt, Pch
Pgestssr359	Supercontig 95: 113892-114064	GGGAAAGAACAATGAAAAGTCA	GTCCTCTCCTCCCCCTTCTT	58	173	1	AT	6	2	0.2205	Pch
Pgestssr362*	Supercontig 106: 159562-159757	GAGCGCGAGAGAAAGAAGAA	CTCGCTTCGACCACTAATCC	60	196	2	AG	9	6	0.6061	No Amplicon
Pgestssr364*	Supercontig 107: 19779-19969	ATCAACCATCCGCAAGTCAT	GTCCCTCCCGGTTTCACTAT	60	189	2	AG	6	7	0.8004	Ph
Pgestssr368	Supercontig 114: 26077-26325	CATCTGATCACCGTCACAGC	AGCACAAGCTTCGTTTCTGAG	60	249	7	AAC	7	4	0.4524	No Amplicon
Pgestssr369*	Supercontig 115: 108439-108655	TCCGGTAAGAGGACAACCAC	CGGTTGTGGTTGGAGAGAAT	60	224	8	ATG	6	5	0.5724	No Amplicon
Pgestssr378*	Supercontig 131: 8697-8937	TGGATGAATCCCGATAATGG	GGTTCGAAGGGTGTTGAGAT	60	241	6	AAG	6	5	0.6818	Pt, Ph
Pgestssr379*	Supercontig 131: 94335-94696	GCATCGGGGGAAAGAAAT	ATTCCGAGGAGTCGGATGAT	60	290	6	AAG	8	8	0.8025	No Amplicon
Pgestssr380*	Supercontig 137: 102093-102252	TCACCCTCAACCTCAACCTC	GACTGGATTGGATTGGATGG	60	163	11	AGC	6	6	0.6176	Ph, Pch
Pgestssr381*	Supercontig 147: 16727-16968	TGAGAACACATCAACACAACTCA	ACCAACCCATTCCTACATCG	59	242	6	AAG	6	5	0.6755	No Amplicon
Pgestssr384*	Supercontig 159: 26223-26376	AATTCCGGGGCAATCTATTC	TATAACGCCCACTCCAGCTT	60	154	3	AC	6	3	0.3599	Pt, Ph

[a] The * indicates the locus showing polymorphism between isolates 7A and 4A.

[b] The location of each SSR locus was determined by BLASTN analysis of the corresponding EST sequence against the 392 supercontigs of the draft genome sequence of *P. graminis* f. sp. *tritici*. The matched supercontig and the start and end base pair coordinates aligned by the two primers are indicated. Pgestssr273 matched to Suppercontig 47 in a region where sequence was missing.

[c] Tm was calculated based on the primer sequence. However, an annealing temperature of 52°C was used in the polymerase chain reactions (see Materials and Methods).

[d] The size was based on the distance between the two flanking primers in the EST sequence.

[e] The 14 microsatellite classes are as follows: 1 = AT/TA; 2 = AG/GA/CT/TC; 3 = AC/CA/TG/GT; 4 = GC/CG; 5 = AAT/ATA/TAA/ATT/TTA/TAT; 6 = AAG/AGA/GAA/CTT/TTC/TCT; 7 = AAC/ACA/CAA/GTT/TTG/TGT; 8=ATG/TGA/GAT/CAT/ATC/TCA; 9 = AGT/GTA/TAG/ACT/CTA/TAC; 10 = AGG/GGA/GAG/CCT/CTC/TCC; 11 = AGC/GCA/CAG/GCT/CTG/TGC; 12 = ACG/CGA/GAC/CGT/GTC/TCG; 13 = ACC/CCA/CAC/GGT/GTG/TGG; and 14 = GGC/GCG/CGG/GCC/CCG/CGC.

[f] Polymorphism information content (PIC) was calculated using the online program at http://www.liv.ac.uk/~kempsj/pic.html.

[g] The primer pairs amplified in the other rust species are indicated with Pt for *P. triticina*, Ph for *P. hordei* and/or Pch for *P. coronata* f. sp. *hordei*, respectively. The primer pairs attempted but no PCR products generated are marked with "No amplicon". The primer pairs not evaluated for cross-species amplification is denoted with a "–".

Therefore, even if the preliminary screening of the primers were done with the DNA of isolate 7A as template, many of the primers would still not amplify. The low efficiency and specificity in PCR amplification for EST-SSR loci were also observed by Dracatos et al. (10) who showed only 12 of 55 primer pairs evaluated in *P. coronata* f. sp. *lolli* successfully amplified DNA fragments in this species. It appeared that the success rate of efficient primers for rust fungi with dikaryotic nuclei is lower compared to the haploid ascomycete fungi. This is probably due to the fact that rust fungi generally have larger genome sizes and more complex genome structures, such as a higher frequency of repetitive DNA sequences and higher DNA polymorphism within a single cell.

With the draft genome sequence available, we were able to localize the 72 SSR loci in 72 different contigs and 53 of the 392 supercontigs assembled from the genome sequences of *P. graminis* f. sp. *tritici*. Knowledge of their locations in the genome is important for population genetic studies because if markers clustered in the same region were selected for a genetic diversity analysis, the measurement of gametic disequilibrium might be inappropriate or biased. Using a mapping population derived from the cross between isolates 4A and 7A, a partial genetic linkage map was developed, which consisted of mostly AFLP and RAPD markers along with avirulence loci (36). The 99 SSR markers polymorphic between the two parental isolates (4A and 7A) provide additional markers for the genetic linkage map. The saturation of the genetic map with these EST-SSR loci will not only assist in map-based gene cloning but also facilitate the assembly of the whole genome sequence. For example, if EST-SSR loci from different supercontigs are found linked to the same linkage group, the supercontigs can be assigned to the same chromosome.

Twenty-five SSR markers were developed from the SSR-enriched genomic DNA libraries of *P. graminis* f. sp. *tritici*, but none of them were amplified in other rust fungi evaluated (32). Our study showed that many of the EST-SSR primers from *P. graminis* f. sp. *tritici* generated amplicons from three other rust species tested, including *P. hordei*, *P. triticina*, and *P. coronata* f. sp. *hordei*. The high transferability of EST-SSRs has been observed in other fungal species. For example, Goodwin et al. (11) tested 99 primer pairs designed from the *M. graminicola* EST database on the closely related species *Septoria passerinii* and found that 66% of them amplified. Eight of twelve primer pairs tested also amplified on the more distantly related species *M. fijiensis* (11). Dracatos et al. (10) used 55 primer pairs for EST-SSR loci of *P. coronata* f. sp. *lolii* to amplify the DNA from various fungal (*P. cronata* f. sp. *avenae*, *P. striiformis* f. sp. *tritici*, *Neotyphodium lolii*, *Blumeria graminis*, *Aspergillus nidulans*, and *Penicillium marneffei*) and plant (*Lolium perenne*) species and had a success rate of amplification ranging from 22 to 53%. This suggests that SSR primers developed from EST sequences may be more easily transferred to other related species. The more closely related the organisms, the higher the rate of the transferability due to more closely related species sharing more homology in SSR-containing genes. High transferability of EST-SSR markers is one of the advantages over the method of generating SSR markers derived from non-coding genomic regions.

Our results indicate that *P. graminis* f. sp. *secalis* is very closely related to *P. graminis* f. sp. *tritici* since most of the 72 primer pairs amplified in the four *P. graminis* f. sp. *secalis* isolates. Interestingly, the four *P. graminis* f. sp. *secalis* isolates didn't form a distinct cluster different from the *P. graminis* f. sp. *tritici* isolates based on the 72 EST-SSR loci. Two of the rye stem rust isolates were indistinguishable from several of the wheat stem rust isolates (Fig. 3). This result further confirms those of Burdon and Marshall (3) and Anderson and Pryor (2) who demonstrated that these two formae speciales were very closely related to each other. Burdon et al. (4) also showed that some virulent isolates could be derived from somatic hybridization of these two formae speciales and were confirmed using isozymic and RFLP markers.

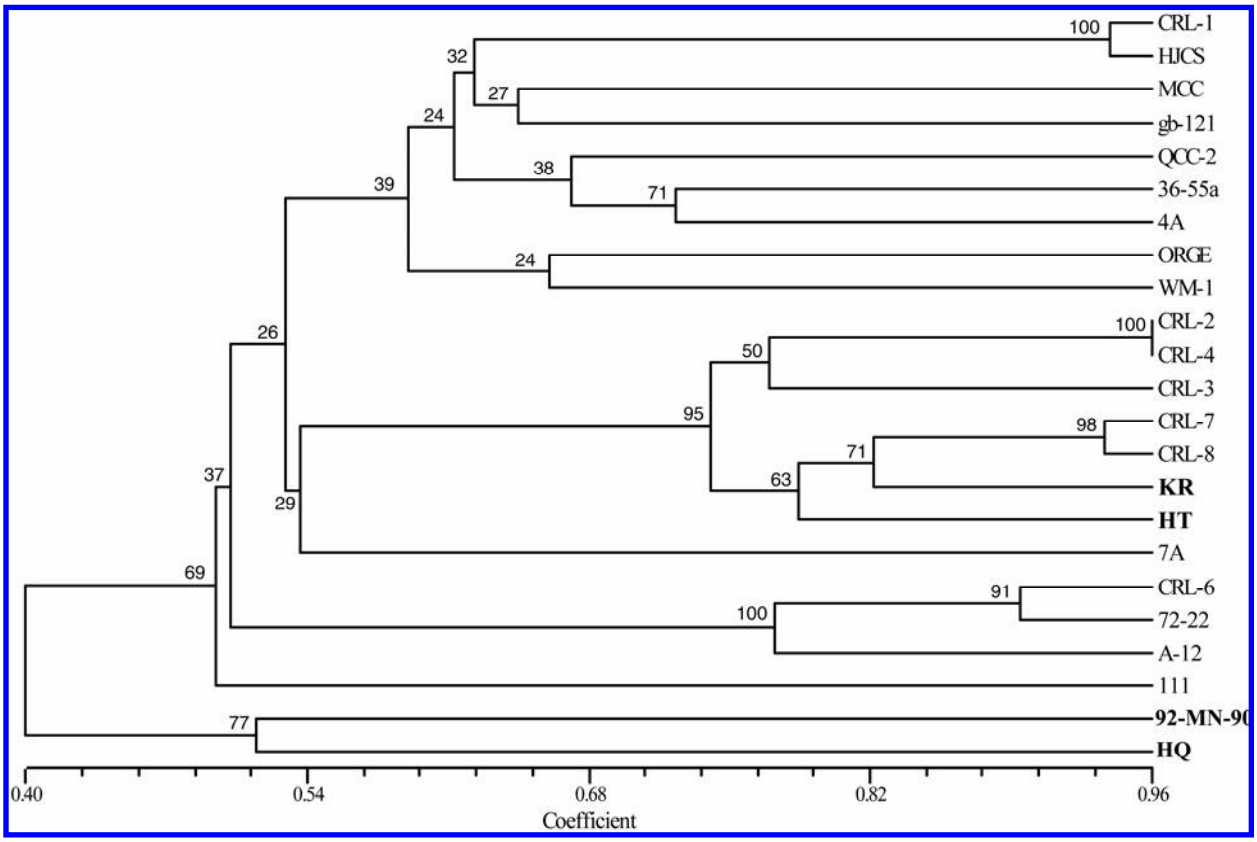

Fig. 3. A dendrogram of 19 isolates of *Puccinia graminis* f. sp. *tritici* and four isolates (bold) of *P. graminis* f. sp. *secalis* generated by cluster analysis with the unweighted pair group method with arithmetic averages. A total of 359 alleles from 72 simple sequence repeats (SSR) loci were used in the analysis. The numbers at the nodes of major clusters represent bootstrap values generated by 500 replications using the Winboot program (25).

The two rye stem rust isolates KR and HT were not only virulent on rye plants but also infected most of the wheat stem rust differentials (*unpublished data*). Therefore, further investigation is needed to reveal the relationship of these rye stem rust isolates with the wheat stem rust isolates.

Roelfs et al. (28) showed that the American isolates could be separated into eleven clusters based on similarity in isozymic and virulence loci. In general, our cluster analysis of isolates was in agreement with those of Roelfs et al. (28). For example, QCC-2, a race that attacked barley with the resistance gene *Rpg1*, was separated from other North American isolates. Several other clusters in our study correspond to those of Roelfs et al. (28), including CRL-8 (T_ _K), CRL-3 (R_ _Q), CRL-1 (Q_CQ), MCC (MCC_), CRL-6 (RCRS), and 72-22 (RHTS). It is notable that CRL-6 (RCRS) and 72-22 (RHTS) are very closely related and probably should be considered the same cluster. These two isolates were also very similar in the analyses of Burdon and Roelf (7) and Roelfs et al. (28) although they were separated into two different clusters by these authors. The isolates 4A and 7A were from the sexually reproducing North American population of *P. graminis* f. sp. *tritici* (36); they were separated from other isolates derived from asexually reproducing populations except for isolate 36-55a which showed some relationship with isolate 4A. Isolate 36-55a was collected in 1936 (A. Roelfs, *personal communication*) and was probably derived from a sexually reproducing population due to the barberry plants not being completely eradicated at that time.

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